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STABILITY OF ASCORBIC ACID IN NEERA FROM DATE PALM (*PHONIX SILVERSTRES*)

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(Communicated by Dr. Mata Prasad, F.A.Sc.)

In a previous communication[1] it has been shown that ascorbic acid in neera was stable even on storage at 37° C. for 15 days. This is an interesting observation since ascorbic acid is known to be unstable in biological materials. The unstability of ascorbic acid in biological materials has been shown to be due to the presence of ascorbic acid oxidase.[2, 3, 4, 5, 6, 7] The present investigation was undertaken with a view to study the nature of different factors which might be present in neera and which might protect ascorbic acid against oxidation.

The samples of neera used in the investigation were collected in the suburbs of Bombay and brought to the laboratory in an ice-cold condition where they were preserved in the refrigerator. No preservative was added to these samples. To study the effect of storage on the ascorbic acid content, the different samples were kept in the incubator and aliquots were taken out at known intervals of time. Ascorbic acid was estimated by the titremetric method of Birch, Harris and Ray[8] (using 2-6 dichlorphenol indophenol).

The ascorbic acid content of some 20 samples together with the effect of storage at 37° C. for various intervals of time was studied. The values for 5 samples representatives of 20 samples studied are given in Table I.

It will be seen from the table that the different samples of neera show a great variation in their ascorbic acid content and the loss of ascorbic acid on storage at 37° C. for 72 hours is not very large.

The stability of ascorbic acid in neera might be due to:

(1) Acidity of the test solution. During storage at 37° C. for 72 hours the pH of neera changed from 7 to about 3. The stability of ascorbic acid could not be ascribed to the high acidity which developed on storage was shown by incubating pure ascorbic acid at 37° C. for 72 hours with acetic acid. Under the conditions of the experiment only a very small retention of ascorbic acid was obtained.

TABLE I

Stability of Ascorbic Acid in Neera on Storage at 37° C.

Sample groups	Average ascorbic acid mg./100 c.c. neera	Time of storage in hours (P.C. ascorbic acid retained) average			
		0	24	48	72
I	7·3	100	95·67	92·9	90·84
II	9·56	100	86·28	81·19	73·33
III	10·27	100	93·29	91·37	88·1
IV	11·92	100	97·22	95·86	95·46
V	12·93	100	98·76	86·43	85·07

(2) The presence of protective factors or to the presence of inhibitors of ascorbic acid oxidase; and

(3) the presence of dehydro-ascorbic acid reductase.

The second possibility of stabilisation due to the presence of inhibitor of ascorbic acid oxidase in neera was tested by incubating pure ascorbic acid with the oxidase in the presence and in the absence of neera. An aqueous extract of coriander leaves was used as the source of the oxidase. About eight samples of neera were used for the experiment, which was set up together with the proper controls as described in Table II. The different solutions were incubated at room temperature (29° C.) for two hours and then made upto 100 c.c. with 5% acetic acid. Aliquots of 5 c.c. were used for titration against the standard dye solution. The results are given in Table II.

It will be seen from the table that:

(1) Ascorbic acid is almost completely oxidised by the oxidase of coriander leaves;

(2) The oxidation of ascorbic acid by the oxidase is inhibited when neera is included in the reaction mixture thereby indicating the presence of an inhibitor.

It has been shown by Mawson,[8] Hopkins and Morgan,[9] Borsook, Devenport, Jeffreys and Warner[10] and Crook and Hopkins[11] that sulfhydryl compounds prevent aerobic oxidation of ascorbic acid, in other words these compounds act as inhibitor of the ascorbic acid oxidase. In view of this observation it was felt to be of interest to see if sulfhydryl compounds are present in

TABLE II

The Effect of Addition of Neera on the Oxidation of Ascorbic Acid by the Oxidase

	c.c. of dye solution for 5 c.c. reaction mixture								
	1	2	3	4	5	6	7	8	9
c.c. of ascorbic acid	10	10	10	..	10	10	..	..	..
c.c. of H_2O ..	27	25	25	12	2	..	10	10	35
c.c. of oxidase extract	..	2	..	..	..	2	2	..	2
c.c. of oxidase extract (boiled)	..	..	2	..	..	..	..	2	..
c.c. of Neera ..	..	..	..	25	25	25	25	25	..
Samples ..									
1 ..	0·70	0·11	0·59	1·45	2·15	2·15	1·45	1·45	..
2 ..	0·63	0·10	0·63	0·84	1·47	1·47	0·84	0·84	0·05
3 ..	0·57	0·10	0·55	1·20	1·77	1·77	1·14	1·14	0·05
4 ..	0·63	0·11	0·60	1·00	1·63	1·58	0·84	1·00	0·05
5 ..	0·67	0·10	0·61	1·55	2·23	2·07	2·12	1·55	0·05
6 ..	0·53	0·05	0·50	1·26	1·9	1·85	1·26	1·26	0·05
7 ..	0·60	0·05	0·55	1·15	1·75	1·75	1·15	1·15	0·05
8 ..	0·54	0·05	0·50	1·35	1·90	1·80	1·29	1·35	0·05
Effect of storage Time of storage in hours									
I 0 ..	0·63	0·10	0·61	0·61	1·47	1·47	0·84	0·84	0·05
24 ..	..	..	..	..	..	..	..	..	..
48 ..	0·63	0·10	0·61	0·68	1·30	1·26	0·63	0·63	0·05
72 ..	0·63	0·10	0·61	0·65	1·28	1·15	0·60	0·60	0·05
II 0 ..	0·67	0·10	0·60	1·20	1·87	1·87	1·14	1·14	0·05
24 ..	0·67	0·11	0·60	1·10	1·77	1·74	..	1·10	0·05
48 ..	0·67	0·10	0·60	1·00	1·70	1·55	0·98	0·98	0·05
72 ..	0·67	0·05	0·60	1·05	1·75	1·53	1·00	1·00	0·05
III 0 ..	0·63	0·11	0·61	1·00	1·60	1·58	0·94	1·00	0·05
24 ..	0·63	0·11	0·55	0·97	1·60	1·55	0·93	1·00	0·05
48 ..	..	..	..	..	..	..	..	..	..
72 ..	0·63	0·05	0·60	0·95	1·55	1·46	0·86	0·91	0·05
IV 0 ..	0·65	0·10	0·61	1·55	2·23	2·07	1·45	1·55	0·05
24 ..	0·65	0·05	0·61	1·43	2·10	1·94	1·28	1·43	0·05
48 ..	0·65	0·05	0·56	1·41	2·06	1·96	1·41	1·41	0·05
72 ..	..	..	..	..	..	..	..	..	..

neera. When neera samples were tested qualitatively with sodium nitroprusside, a very strongly positive reaction was obtained. Next the concentration of sulfhydryl compounds was estimated by iodometric titration after making a due allowance to the amount of ascorbic acid present as determined by titration against the indophenol dye. The effect of storing neera at 37° C. upto 72 hours on the concentration of sulfhydryl compounds was also studied. The results have been represented as glutathione and given in Table III.

TABLE III

Concentration of Sulphahydryl Compound in Neera mgs./100 c.c.

Samples	Time of storage in hours			
	0	24	48	72
I	89·2	97·8	125·9	130·8
II	116·5	163·2	157·9	169·5
III	130·5	89·7	100·7	109·0
IV	142·4	155·2	166·5	220·8
V	178·9	148·9	159·0	162·1

It will be seen from the table that neera contains a fairly high concentration of sulfhydryl compounds. Hopkins and Morgan[9] have shown that glutathione besides acting as an inhibitor of ascorbic acid oxidase, also accelerates the enzymic conversion of dehydro ascorbic acid to ascorbic acid. Crook[12] isolated the enzyme which he called dehydro ascorbic acid reductase from cauliflower and confirmed the observations of Hopkins and Morgan.

In view of these observations and in view of the high concentration of sulphahydryl compounds in neera it was felt to be of interest to see if dehydro ascorbic acid reductase is present in neera. Two methods were used for the detection of the reductase. The first method followed was essentially the same as described by Crook.[12] Dehydro ascorbic acid was prepared by the oxidation of pure ascorbic acid by an aqueous solution of benzoquinone. McIlvoine's buffer of pH 6·0 was used. The different reaction mixtures were set up as described in Table IV and incubated at room temperature (29° C.) for 10 minutes. They were then made up to volume with 5% acetic acid and 5 c.c. aliquot was titrated against standard dye solution. The results of a typical experiment are given in Table IV.

TABLE IV

Detection of Reductase in Neera

Numbers	Reaction Mixture	c.c. of dye required
1	3 c.c. buffer + 1 c.c. DHAA + 1 c.c. water	0·15
2	3 c.c. buffer + 1 c.c. DHAA + 1 c.c. neera	2·1
3	3 c.c. buffer + 1 c.c. DHAA + 1 c.c. Neera (boiled)	1·35
4	3 c.c. buffer + 1 c.c. water + 1 c.c. Neera	1·2
5	3 c.c. buffer + 1 c.c. water + 1 c.c. Neera (boiled)	1·2
	Titre value due to conversion of DHAA to AA	0·75

DHAA = *Dehydro-ascorbic acid* AA = *Ascorbic acid*

It will be seen from the table that in the presence of neera dehydro-ascorbic acid is converted to ascorbic acid and this was due to an enzyme.

The second method used for the detection of dehydro ascorbic acid reductase consists in incubating dehydro-ascorbic acid obtained by the oxidation of pure ascorbic acid by the oxidase from coriander leaves with neera and estimating the amount of ascorbic acid formed under the conditions of the experiment. The reaction mixture consisted of 30 c.c. ascorbic acid solution (containing 3 mg. of ascorbic acid) + 6 c.c. of coriander leaf extract. The mixture was incubated at room temperature for 2 hours during which all the ascorbic acid was converted to dehydro-ascorbic acid. It was then divided into three portions, one portion was made upto 100 c.c. with 5% acetic acid and ascorbic acid in 5 c.c. aliquot was estimated. The second portion was incubated with 25 c.c. neera at room temperature for two hours, the third portion was incubated with 25 c.c. neera which was boiled, the volumes of these reaction mixtures were made upto 100 c.c. with 5% acetic acid and the amount of ascorbic acid present in 5 c.c. aliquots estimated. All the other necessary controls were run simultaneously. It was observed on titration that no ascorbic acid was present in 1st portion, the 3rd portion contained ascorbic acid corresponding to that present in neera while the second portion contained ascorbic acid in excess to that present in the corresponding amount of neera. The excess corresponds to ascorbic acid formed by the action of the reductase. Six samples of neera were tested and the amount of ascorbic acid formed by the reductase present in these samples from dehydro ascorbic acid is given in Table V.

TABLE V

Conversion of Dehydro-Ascorbic Acid to Ascorbic Acid

Samples	p.c. conversion
1	90·4
2	125·0
3	90·9
4	71·2
5	14·3
6	39·5

It is clear from the table that all the samples of neera tested contained the reductase which in conjunction with sulfhydryl compounds, *viz.*, glutathione, maintain ascorbic acid in the reduced form.

SUMMARY

1. Ascorbic acid in neera was estimated by titration against 2:6 dichlorophenol indophenol. The samples showed a marked variation in ascorbic acid content.

2. The loss of ascorbic acid in neera was very small on storage at 37° C. for 72 hours.

3. A high concentration of sulfhydryl compounds was observed in neera.

4. Presence of dehydro-ascorbic acid reductase has been shown in neera.

5. The stability of ascorbic acid in neera has been attributed to the presence of (i) sulphahydryl compounds and (ii) dehydro-ascorbic acid reductase.

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THE DEVELOPMENT OF *HERDMANIA PALLIDA* (HELLER)

By V. O. Sebastian

(From the University Zoology Research Laboratory, Madras)

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(Communicated by Prof. R. V. Seshaiya)

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Introduction

The present paper is the result of the researches conducted by the author in the years 1937–40, on the Ascidians of the Madras coast, especially on

development and larval organisation, in partial fulfilment of the requirement for the Degree of Master of Science. The work remained unpublished till now, except for a brief account which was read before the annual session of the Indian Science Congress held at Lahore (Sebastian, 1939). The existing literature on the Tunicates of India is confined only to recording of species and anatomy, done at very wide intervals (Bomford, 1913; Herdman, 1891, 1906; Oka, 1915; Sewell, 1926; Menon, 1931; Das, 1936, 1938, 1940, 1945; Sebastian, 1942; Nair and Aiyer, 1943; Nair, 1949). The special features of development characteristic of *Herdmania pallida* living in the tropical sea at Madras should be interesting.

This work is of additional interest on account of the peculiar position which the family Pyuridæ holds in deciding the phylogeny of ascidians (Berrill, 1948 *a*). So far, among the Pyuridæ, the developmental stages of only *Halocynthia pyriformis* (Rathke), *Boltenia echinata* (L.), and *Pyura squamulosa* (Alder) are known, that of *H. pallida* being the fourth.

Material and Method

The animals were collected from the Madras harbour, where they live attached to piers, buoys and walls. Inside laboratory tanks they live in healthy condition. *H. pallida* is oviparous and development takes place completely outside the parent in the sea. Developmental stages were obtained both by natural spawning and artificial fertilization, even though Berrill (1950) has mentioned about the difficulty in obtaining fertilized eggs of pyuridæ.

Artificial fertilization was successfully carried out at any time of the day in all months of the year. Healthy animals were cut open and eggs and sperm of different animals mixed in very small quantity of sea-water in finger bowls, and slightly stirred to ensure thorough mixing. Later more water was added and kept for observation. The eggs, on being teased out were found to sink to the bottom of the containers. During the course of development frequent changes of water were made. Even though a good many of the eggs failed to develop, perhaps due to different stages of maturity, sufficiently large number of healthy developing embryos were obtained. The stages from egg to larvæ were examined in the living condition. The larvæ were sectioned for studying the internal organisation.

The temperature of the sea at Madras has a variation from a minimum of 26° C. in the coldest months, to a maximum of 29° C. in the hottest months. The time given for developmental stages is an average of the observations made on various days of experiments.

Bouin's fluid as a fixative was very successful both for whole mounts and sections. Other fixatives like corrosive sublimate and formalin also gave sufficiently good results. Paraffin, and double-embedding in cedukol were used for sections. Stains used were Delafield and Heidenhain's hæmatoxylins.

FERTILIZATION AND CLEAVAGE

The egg is translucent and not heavily yolked, and provided with an outer chorion on which are found rounded outer follicle cells. The ooplasm measures 0·19 mm., and around it are found, scattered inside the chorionic fluid, inner follicle cells or test cells. The development is typical of ascidian eggs (Conklin, 1905). The entrance of the sperm into the egg is marked by movement of the ooplasmic contents. Three zones, *viz.*, the clear cytoplasmic region, the grey yolky region and the crescent of lipoidal inclusions can be made out (Fig. 1). The crescent is not coloured yellow as in the case of *Styela partita* (Conklin, 1905), *Boltenia echinata* (Berrill, 1929) or *Pyura squamulosa* (Millar, 1951). The first cleavage commences 35 minutes after fertilization, and the three zones divide equally (Fig. 2). Gastrulation is embolic, and begins after the sixth cleavage. A deep gastrula is shown in Fig. 3. The time taken from fertilization to gastrulation is 110 minutes, which is comparatively shorter than the time taken by the eggs developing at lower temperatures. Table I gives the comparative time taken by the eggs of *Herdmania pallida* developing at Madras where the sea has a temperature of 26–29° C. with those of *Styela partita* (Conklin, 1905) developing at Woods Hole at a temperature of 22° C.

TABLE I

Time in minutes

Developmental stages	*H. pallida*	*S. partita*
Fertilization to 1st cleavage ..	35	40
1st cleavage to 2nd cleavage ..	10	30
2nd cleavage to 3rd cleavage ..	11	20
3rd cleavage to 4th cleavage ..	15	20
4th cleavage to 5th cleavage ..	18	20
5th cleavage to 6th cleavage ..	21	20
Total time ..	110	150

The difference in the time taken to complete the sixth cleavage is presumably to be attributed to the difference in temperature at the two places.

FIG. 1. Fertilized egg showing the three zones. FIG. 2. The egg after the first cleavage.

The inter-cleavage interval is also not constant as found in the case of *Styela* and *Ascidiella* (Berrill, 1935). The time taken between the first and second cleavage is only ten minutes, and the intervals between the succeeding stages show a regular increase in time. This may be due to the increase in the number of cells after every stage of division.

After gastrulation gradual changes take place within the embryo. The blastopore closes, and the rudiments of the tail begin to appear. An anterior broader and posterior narrower portions are visible, the former being the trunk, and the latter, the tail rudiments (Fig. 4). The notochordal cells

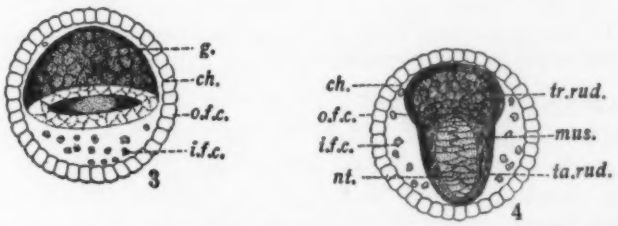


FIG. 3. A deep gastrula. FIG. 4. The stage showing the trunk and tail rudiments.

occupy the central core of the tail. As growth proceeds, they begin to arrange themselves in one row, and the final serial arrangement of cells is reached when the time of liberation approaches. Within the chorion the tail is coiled around (Fig. 5) and shows twitching movements.

LIBERATION OF THE LARVÆ

About 8 hrs. from fertilization the larvæ are liberated by the rupture of the chorion. With the attempt of the larva to straighten its tail, the rupture takes place on the ventral side posteriorly. Just after the tail has straightened out, the chorion can be seen as a cap over the body of the larva, the dorsal region intact and the ventral broken. A few seconds later, the larva wriggles and swims away from the chorion.

The liberation of the free-swimming larva of *H. pallida* within a period of 8 hrs. is very rapid compared with the time of 60 hrs. taken by the eggs of *Halocynthia* and 34 hrs. by *Boltenia* at 16° C. (Berrill, 1935), and about 22 hrs. by *Pyura* at 18-19° C. (Millar, 1951).

THE LARVA

The larva conforms to the urodele type (Fig. 6), and measures 1·15 mm. in length from the tip of the adhesive papillæ to the end of the tail fin, the

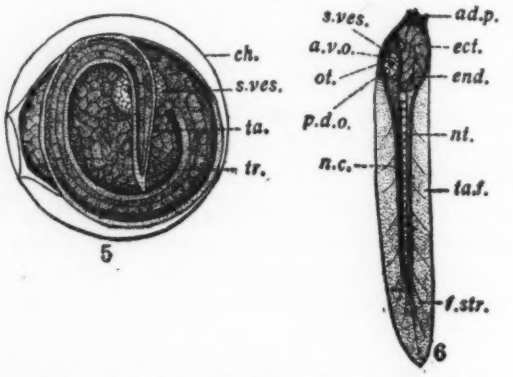


FIG. 5. The stage before hatching. The inner and outer follicle cells are not shown.
FIG. 6. The tadpole larva of *Herdmania pallida*.

trunk measuring 0·30 mm. and tail 0·85 mm. The general organisation is very simple. In the trunk region the dorsal portion is occupied by the sensory vesicle with the contained sensory organs, and the visceral ganglion behind it. Ventral to these is a mass of endodermal cells occupying the rest of the space. There is no trace of peribranchial sacs or siphon rudiments. Rudiments of the heart and pericardium are also absent. In the tail, the central portion is occupied by the rod of notochordal cells, above which is situated the nerve cord. The muscle cells and the endodermal strand occupy the rest of the space.

Tunic.—(Figs. 6, 7 and 9). The body and tail are enveloped by a comparatively thin, non-cellular tunic of glassy transparency. In the region of the tail the tunic is greatly expanded dorso-ventrally and compressed laterally, thus constituting the tail fin. The fin presents slanting lines at regular intervals, with a group of them at closer intervals at the posterior extremity, thus giving a fin-ray-like appearance.

Mantle.—(Figs. 7, 9, 11 and 12). The ectoderm cells forming the mantle cover the whole of the larva. It is of uniform thickness throughout,

gradually thinning to a slight extent in the tail region. It is made up of one layer of large cells, more or less rectangular in shape, possessing very prominent nuclei. Around the nucleus yolk granules are present varying in number in the different cells. They stain deep in Heidenhain's hæmatoxylin. In the living tadpoles they are refractive, but when stained with osmic acid they turn black.

Adhesive papillæ.—(Figs. 6, 8 and 9). They are temporary structures, three in number, constructed on a very simple plan. Each papilla is composed of a group of ectodermal cells of the anterior region of the trunk which have differentiated into secretory cells. They are narrow and elongated

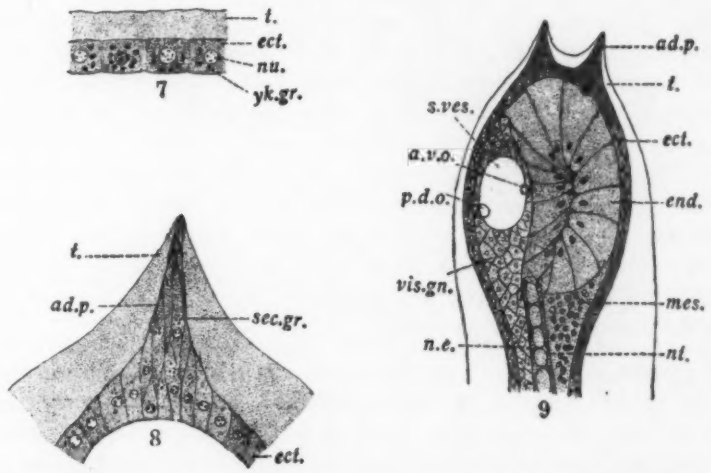


FIG. 7. The mantle (ectoderm) and the tunic. FIG. 8. Adhesive papilla. FIG. 9. Median longitudinal section of the body of the larva (Reconstructed from sections).

with pointed extremities, all packed together into a small conical structure having a height of 45 μ and a basal diameter of 20 μ. The nuclei of these cells are placed towards the base above which are found areas containing products of secretion in the nature of granules. During metamorphosis of the larva, the initial act of fixation is effected by the help of these secretory adhesive papillæ. That the process of fixation is very efficient is seen from the fact that it is only with difficulty that the attached larvæ could be dislodged from their position, by pipetting strong currents of water against them.

Nervous system.—In the larva of *H. pallida*, nervous system shows only the temporary functional organs, *viz.*, the sensory vesicle, the visceral

ganglion and the nerve cord. The sensory vesicle and the visceral ganglion occupy the trunk portion of the larva, and the nerve cord stretches behind in the tail dorsally.

(*a*) *Sensory vesicle.*—(Figs. 6, 9, 10 and 11). This is oval in shape, occupying the trunk region of the larva, the anterior end of which is prolonged into a knob of nerve cells. The cavity of the vesicle is round in transverse and oval in longitudinal sections. Inside this cavity are found the sensory organs, *viz.*, two ocelli and an otolith, which is a unique feature hitherto unrecorded in ascidian tadpoles.

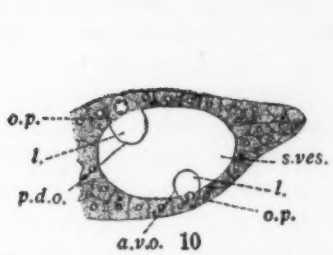
(*b*) *Ocelli.*—(Figs. 6, 9, 10 and Photomicrographs 1 & 3). Of the two ocelli present, one is placed postero-dorsally and the other antero-ventrally. Their structure is very simple (Fig. 10). In sections, it is found that one among the cells that form the wall of the vesicle gets enlarged into an oval shape with a large nucleus at its base. The nuclear portion is embedded in the wall of the vesicle, while the distal portion projects out prominently into the cavity of the vesicle, and possesses a smooth round margin. In stained preparations traces of pigmentation can be seen on the embedded portion. The distal projecting portion having the appearance of a lens also takes the stain, and it is apparently brittle, because it crumbles on contact with the microtome knife. The structure is the same for both the ocelli, the only difference being that the postero-dorsal one is almost double the size of the antero-ventral.

(*c*) *Otolith.*—(Figs. 6, 11 and Photomicrograph 2). This arises obliquely from the ventral wall of the sensory vesicle. The structure is typically unicellular with a comparatively big nucleus situated on the proximal side, and a spherical darkly pigmented body at its distal extremity. The cell is pointed at the distal end and is clearly visible in the living condition, but not when sectioned.

(*d*) *Visceral ganglion.*—(Figs. 9, 11 and Photomicrograph 2). This is the portion behind the sensory vesicle, and in front of the nerve cord. It is composed of large cells with round and prominent nuclei. There is no cavity in its centre.

(*e*) *Nerve cord.*—(Figs. 6 and 12). This lies over the notochord, in the mid-dorsal region of the tail, stretching from the hinder portion of the visceral ganglion to the end of the tail. There is a cavity in the centre extending to the full length.

Endodermal derivatives of the trunk.—(Figs. 6, 9, 11 and Photomicrograph 2). Inside the trunk, the endoderm is found to be very primitive in



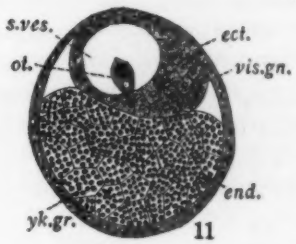


FIG. 10. Longitudinal section of the sensory vesicle (Reconstructed from sections).
FIG. 11. Oblique section passing through the otolith.

organisation. Rudiments of the pharynx, endostyle or intestine are not developed. All that is found is a mass of very large cells, pyramidal in shape, occupying the whole space inside the ectoderm and ventral to the brain vesicle. The broader ends of the cells are placed outwardly touching the ectoderm on the ventral side, and the sensory vesicle on the dorsal margin, and their inner narrower extremities meeting in the centre. Yolk granules are found in such large numbers that they practically occupy the entire space within each cell.

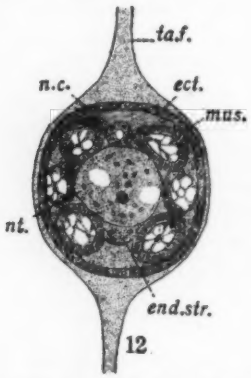
Notochord.—(Figs. 6, 9 and 12). This is very well developed occupying the central region of the tail. A part of the anterior region of the notochord projects into the trunk region touching the posterior side of the endodermal mass of cells, and from here it extends up to the tip of the tail, below the nerve cord. It is composed of a single row of vacuolated, turgid, cylindrical cells placed one behind the other. Fatty granules are found inside each cell. Due to the rigidity of the notochord the tail only bends in arcs while swimming.

Endodermal strand.—(Fig. 12). Ventral to the notochord and placed between the edges of the lower muscle bands of the tail are granulated endodermal cells forming the endodermal strand.

Mesenchyme cells.—They are found scattered in different regions within the space between the outer ectoderm and internal organs. A thick mass of them is found behind the posterior portion of the endoderm of the trunk where the latter is in contact with the anterior end of the notochord (Fig. 9).

Tail muscles.—The structure of the muscle band is clearly made out in transverse sections of the tail region (Fig. 12). There are two bands, each one composed of three parallel rows of muscle cells applied to the side of the notochord. Towards the posterior extremity of the tail only two rows of muscle cells are found, the middle row being absent. Each muscle cell

has a darkly staining cortex and a clearer central area. The cortex shows striated nature which is due to the dark and lightly staining regions of the protoplasm alternating with one another. The dorsal and ventral rows of muscle cells show striations uniformly around their edges, while on the middle row they are confined only to the sides adjoining the notochord. The central non-striated area of muscle cells is a vacuolated space, the nucleus held in position by cytoplasmic strands.



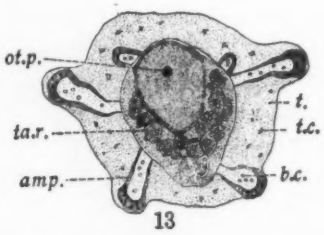


FIG. 12. Transverse section of the tail of the larva. FIG. 13. Post-larval stage.

BEHAVIOUR OF THE LARVA

During the early part of the free-swimming life the tadpoles swim rapidly towards the surface, the anterior part directed upwards, the tail moving in arcs. They show a preference for swimming around the edge of the vessel. Sometimes the larva hangs perpendicularly with the adhesive papillæ in contact with the surface of water, and swims in small circles, at the same time rotating on its own axis. Generally, after half to one hour of active life, the larvæ become inactive, only swimming at intervals, and they are found to drop to the bottom of the vessel attaching to the substratum they come across. Even though no detailed experiments were conducted on the behaviour of the larvæ, the early geo-negative (photo-positive) and later geo-positive (photo-negative) tendencies shown are not unlike that found in the larvæ of *Amaroucium*, *Molgula* and *Botryllus* tadpoles described by Grave (1920, 1926), Mast (1921) and Grave and Woodbridge (1924).

POST-LARVAL STAGE

The free-swimming period of the larva of *H. pallida* lasts, generally, for 3 to 3½ hours after which it fixes on the substratum by means of the adhesive papillæ. Exceptionally, larvæ have been found to be active for about

12 hours. After fixation, metamorphosis is marked by the contraction of the tail. The contents of the latter break up into small bits and are resorbed, the resorption beginning from the posterior end, until finally the outer cuticle of the tail remains for sometime as a hollow tube. Simultaneously with the break-up of the tail, ectodermal outgrowths of the trunk, or the ampullæ begin to appear. Their growth is accompanied by the outpushing of the test which spreads out and firmly fixes the metamorphosed larva. Apart from the minor role of fixation which the ampullæ serve, they have a very important part to play in respiratory activity. In the living condition careful observation shows corpusculated fluid flowing in and out of the ampullæ when the heart reverses the course of circulation, which is indicative of respiratory function.

There are six ampullæ (Fig. 13 and Photomicrograph 4), three on each side, two very short and four longer ones. The short ones are 10 μ and longer 40 μ in length. The distal extremities of all have a width of about 20 μ. The walls of the ampullæ are made up of more or less cubical cells with spherical nuclei. These cells tend to become columnar as they approach the distal ends, with nuclei placed towards their basal half. In the newly fixed post-larva the body takes an oval shape, the long axis measuring 214 μ, and the shorter 132 μ. The otolith persists in the just fixed stage and can be seen prominently.

General

Millar (1951) from his observations on the development of *Pyura* considers its larva to be more primitive than that of either *Boltenia* or *Halocynthia*. The tadpole of *Herdmania* is nearer to that of *Pyura* in the absence of peribranchial invaginations or siphon rudiments. But, the single-celled nature of the ocelli makes it even more primitive than the tadpole of *Pyura*, and in the possession of two independent ocelli tadpole of *Herdmania* stands apart from all known ascidian tadpoles.

Discussion

The three independent sensory organs of the tadpole of *H. pallida*, two ocelli and an otolith, are of outstanding importance in deciding the polyphyletic origin of ascidians. The normal sensory equipment so far known in ascidian tadpoles, is the otolith and the ocellus. The otolith, if present, is identical in all cases, being unicellular. The ocellus has a complex structure with three lens cells, an optic pigment-cup and about a dozen retinal cells. In the great majority of Enterogona this normal equipment is the rule, even though there are exceptions like the tadpole of *Pycnoclavella* without an otolith, and of *Polycitor* without an ocellus (Berrill, 1947 and 1948 *b*).

In Pleurogona more types of variations are met with, like the diminished ocellus in *Styela*, combined organ or photolith in Polystyelids, and absence of ocellus in Molgulids. Grave (1944), from his observations on the nature of the diminished ocellus of *Styela* and the combined organ of Botryllids, has maintained that ascidians are polyphyletic in origin. Berrill (1948 *a*) has contradicted Grave's view on the ground that variations in the structure of the sensory organs are only deviations from the normal type due to degeneration resulting in diminution, combination or disappearance. He also maintains that, " The best developed and least specialised family is the Pyuridæ, with less known about its larvæ than any other ascidian family. The question whether there has been a multiple origin of the tadpole, or whether the pleurogonid tadpoles are modifications of the enterogonid, is most likely to be settled by more knowledge of this family". Berrill's statement must carry weight. The tadpole of *H. pallida*, with its two independent, unicellular ocelli, definitely points to a polyphyletic origin in ascidians. Yet, there remains the question of standard sensory equipment of ascidian tadpoles.

If the tadpole of *H. pallida* is the least specialised one, the sensory equipment is also to be considered as the most primitive. Taking the otolith, it is found to be not different from that found in any other ascidian tadpole. The problem arises only in the case of the ocelli. One departure from others is that the ocelli are two in number, and independent of each other. The second difference is the unicellular nature, which is unlike the complex structure of ocellus met with in all known forms. From this it is to be presumed that the complex ocellus is a later development from the type of primitive unicellular, independent ocelli met with in forms like *Herdmania* due to fusion, and that, still later in the process of evolution, further degeneration occurred resulting in diminution, combination or disappearance. But, if by fusion an ocellus with three lens cells is to be formed, it is only reasonable to suppose that the primitive ascidian tadpole should possess at least three independent unicellular ocelli. In that case there is the necessity to think of a still more primitive ascidian tadpole than that of *H. pallida*. Hence, the following suggestion is made to explain the probable course of evolution of the sensory equipment of ascidian tadpole. ***This suggestion is only tentative.***

A hypothetical ascidian tadpole is to be imagined, which is the most primitive one, and possessing three independent, unicellular ocelli, and a unicellular otolith. While the otolith of such a tadpole has remained unchanged during the course of evolution, the ocelli have fused to form a complex ocellus with three lens cells and associated structures, and the latter,

again undergoing modification due to secondary degeneration changes. There may also be a different explanation. The ocelli can be expected to have undergone modifications through two different lines. One is the way described above, *i.e.*, primary fusion to form a complex ocellus, and secondary degeneration. The other is, degeneration of one or more of the independent ocelli without undergoing a primary fusion, resulting in certain cases, complete disappearance, the otolith alone remaining. The tendency towards such degeneration is indicated in the diminished nature of one of the ocelli of *H. pallida.*

Summary

1. The development of *Herdmania pallida* (Heller) is described. Artificial fertilization was very successful resulting in healthy embryos.

2. The egg is small, and not heavily yolked. Gastrulation is embolic. The larva is liberated in 8 hours. The free-swimming period lasts for 3 to 3½ hours. During fixation, six ampullæ are developed, of which two are very short, the other four being considerably longer.

3. There are three sensory organs inside the sensory vesicle, *viz.*, two unicellular ocelli of which one is small, and an otolith. This is the first record of the occurrence of three sensory organs inside the sensory vesicle of an ascidian larva.

4. The primitiveness of organisation of the tadpole, and the nature of the sensory organs, point to a polyphyletic origin in ascidians.

5. The question of the standard sensory equipment of ascidian tadpole is discussed. A tentative suggestion is made about the probable course of evolution of ascidian tadpoles, based on the structure of sensory organs.

Acknowledgements

Grateful acknowledgements are made to Prof. R. Gopala Aiyer, the former Director of the University Zoology Research Laboratory, Madras, under whose guidance the work was carried out; and also to the Madras University, for awarding me a Research Studentship. I am also thankful to Prof. R. V. Seshaiya, for kindly going through the manuscript and forwarding it to the press.

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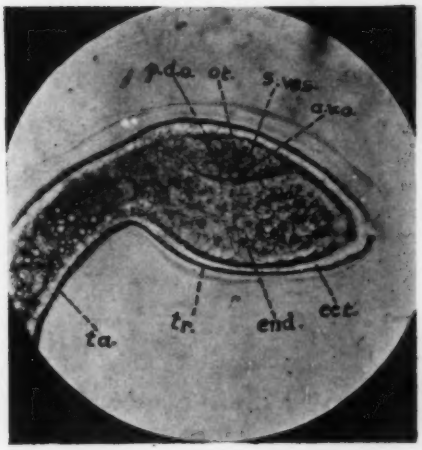
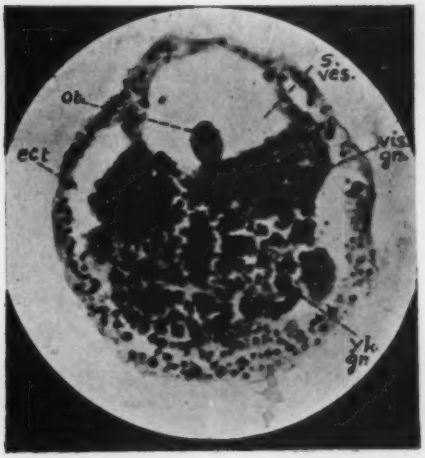
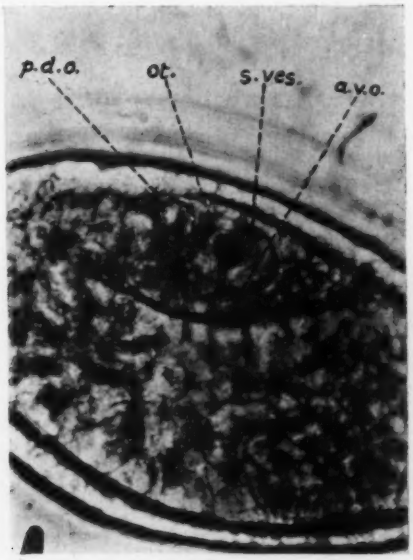


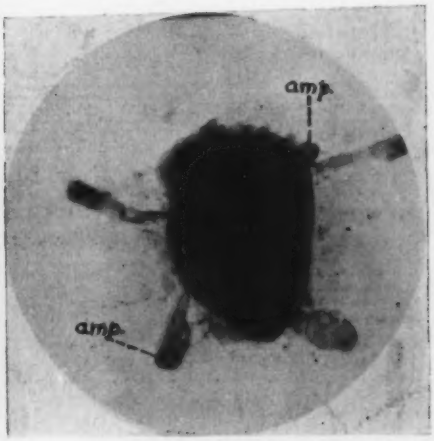
1. Photomicrograph of the body and portion of the tail of the living larva.



2. Photomicrograph of the section passing through the otolith.



3. An enlarged photomicrograph of the body of the larva.



4. Photomicrograph of a just fixed post-larva.

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Key to Lettering

ad.p., Adhesive papillæ ; *amp.*, Ectodermal ampullæ ; *a.v.o.*, Antero-ventral ocellus ; *b.c.*, Blood corpuscles ; *ch.*, Chorion ; *cl.cy.*, Clear cytoplasm ; *cr.*, Crescent ; *ect.*, Ectoderm ; *end.*, Endoderm ; *end. str.*, Endodermal strand ; *f. str.*, Striations of the tail fin ; *g.*, Deep gastrula ; *gr. yk.*, Grey yolk ; *i.f.c.*, Inner follicle cells ; *l.*, Projecting lens-like portion of the ocellus ; *mes.*, Mesenchyme cells ; *mus.*, Tail muscle ; *n.c.*, Nerve cord ; *nt.*, Notochord ; *o f.c.*, Outer follicle cells ; *o.p.*, Embedded part of the ocellus with traces of pigmentation ; *ot.*, Otolith ; *ot. p.*, Pigmented body of the otolith ; *p.d.o.*, Postero-dorsal ocellus ; *s. ves.*, Sensory vesicle ; *sec. gr.*, Secretory granules ; *t.*, Tunic ; *ta.*, Tail ; *ta. rud.*, Tail rudiment ; *ta. r.*, Broken bits of tail tissues undergoing resorption ; *t.c.*, Cells of the outer test ; *tr.*, Trunk ; *tr.rud.*, Trunk rudiment ; *vis. gn.*, Visceral ganglion ; *yk. gr.*, Yolk granules.

ROLE OF ACTOMYOSIN IN CONTRACTION OF UNSTRIATED MUSCLE

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Unstriated muscle contains actomyosin (Rosza, 1946; Lajtha, 1947; Csapo, 1948, 1950). The effect of substances on the contractile mechanism of unstriated muscle has been studied by using the dying muscle (Singh, 1943 *a*; 1944 *a*; Singh and Singh, 1949, 1950 *a*, 1950 *b*, 1951). The muscle is immersed in the experimental solution, its oxygen supply is stopped and is allowed to die therein. It is well known that the normal permeability of the muscle depends upon metabolism, so that as the muscle dies, it becomes inexcitable as well as fully permeable to the substance; the excitatory system is then knocked out and the contractile system acts without interference. The time adopted for immersion has been 24 to 48 hours (and even 72 hours), but it has been found that 24 hours are usually sufficient, there being no significant difference between the effect of immersion for 24 hours and longer periods. In 48 hours, the muscle invariably dies, but after 24 hours some of these muscles may not die and may actually recover after suitable treatment. The results from the latter muscles may undoubtedly be said to represent the physiological behaviour of the muscle. Besides the dying muscle, the inexcitable muscle may be used, the muscle previously having been rendered inexcitable by methyl alcohol and high voltages (Singh and Singh, 1951); in the present experiments chloroform has been used.

At present, there is a wide gap between the physiologically active muscle on one hand and Szent Gyorgyi's extracted muscle and actomyosin solution on the other; the dying muscle is a link between these preparations. Thus we have various preparations, which may be graded according to their physiological condition as follows:—Normal physiologically active muscle, inexcitable but otherwise physiological muscle, dying muscle which can be restored to physiological condition, dying muscle which ultimately dies, extracted muscle (Szent Gyorgyi), actomyosin threads (H. H. Weber) and actomyosin solution (Szent Gyorgyi).

During experiments on dying muscles some remarkable resemblances between the action of ions on muscle and on actomyosin in solution were noted. Thus substances, which cause the dissociation of actomyosin, cause unstriated muscle to relax. There are two groups of these substances

which cause dissociation of actomyosin, salts on the one hand and urea on the other. It was therefore considered advisable to list the effect of those substances, the action of which on the association or dissociation of actomyosin is known, on the contraction or relaxation of dying unstriated muscle. A close parallelism has been found and it appears that (1) one kind of contraction of unstriated muscle is due to association of actomyosin, (2) passive relaxation of unstriated muscle is due to the kind of dissociation of actomyosin produced by salts, (3) active relaxation of unstriated muscle is due to the kind of dissociation of actomyosin produced by urea, (4) superprecipitation of actomyosin corresponds to contraction.

Experimental

Dogs were anæsthetised with chloroform and frogs killed with a blow on the head; their stomachs were removed and opened at the lesser curvature. Transverse pieces were taken, their mucosa removed and the pieces bisected at the greater curvature. Ligatures were tied at the ends of one of the two pieces, and the other piece was left untied. They were oxygenated for half an hour in saline. By such procedure they reached a constant length, which was measured, the length of the ligatured piece being measured between the ligatures; this may be termed as length A. Pieces near the cardiac and pyloric ends should not be used, as they do not contract properly.

Two sets of experiments were then performed; in one the ligatured pieces were suspended and loaded with 7–8 g. and in the other the unligatured pieces were left unloaded to test passive and active relaxation respectively. The technique for studying active relaxation has been described previously (Singh and Singh, 1949, 1950 *b*). If both sets of pieces were ligatured, then one set was used as a control.

When the muscles were loaded, they inevitably lengthened, and with the small load used, they reached a constant length in an hour's time. Their length was again measured; this may be termed as length B. After immersion in various solutions, they contracted or relaxed due to the action of the excitatory system. As they were dying, after some hours, they began to readjust themselves and reached new lengths due to the action on the contractile mechanism. After 24 hours' immersion, the length was measured (this may be termed as length C) and expressed as p.c. of the inittial length of the muscle before loading, that is, length A. Some muscles relaxed more and the others less in the same experiment and for comparison, the length in distilled water was taken as the standard; muscles with shorter length were considered to be relatively contracted and those with greater length, relatively relaxed.

For purposes of calculation, the length B can be taken into consideration. The results ran parallel in the two methods, but were not so regular in the second method, owing to the presence of spontaneous contractions and thus varying the length B. In these experiments, therefore, the 1st method has been adopted and the calculations are based on the lengths A and C.

These experiments were performed at 20° C. At higher temperatures, the question of bacterial action arises, so that at these temperatures, the control muscles were treated with solutions, saturated with chloroform.

RESULTS

Effect of potassium.—In 24 experiments, loaded pieces of dog's stomach muscle were suspended at 20–30° C. in various concentrations of potassium chloride, starting from 0·1 *M* to 0·6 *M* KCl, increasing in steps of 0·1 *M*. It was found that maximum contraction occurred in 0·1 *M* KCl in 20 experiments (Fig. 1), in 0·1–0·3 *M* KCl in 2 experiments and only relaxation in

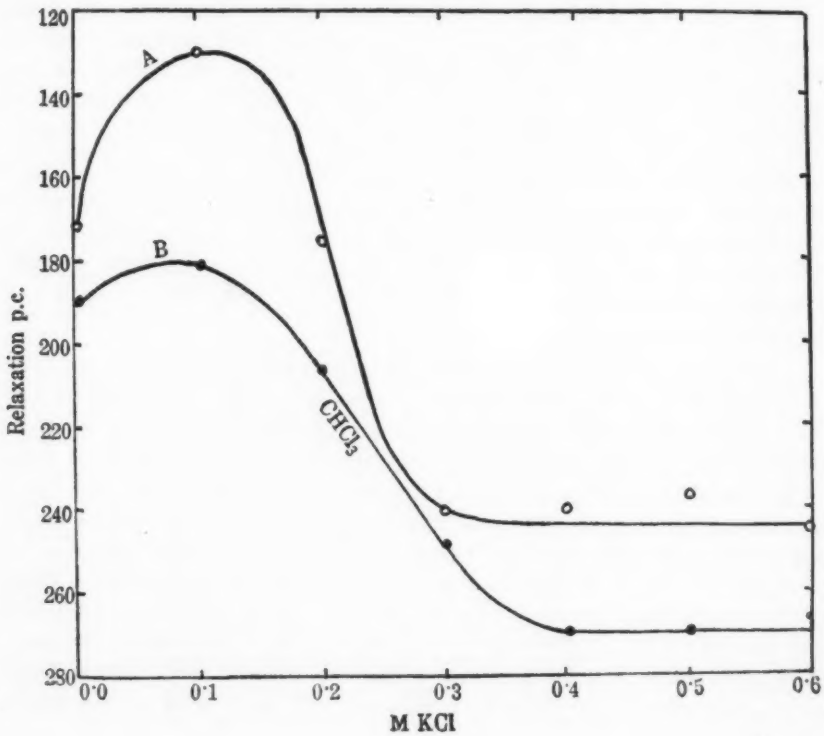

FIG. 1

FIG. 1. Dog's stomach muscle. Effect of potassium chloride on contraction and relaxation. 24 hours' immersion without oxygen.

A. Upper curve in potassium solution.

B. Lower curve. Same solutions as in A, but saturated with chloroform.

2 experiments (Fig. 2). In 4 experiments on frog's stomach muscle, maximum contraction occurred in 0·1 *M* KCl, and in 2 experiments, in 0·1–0·3 *M*

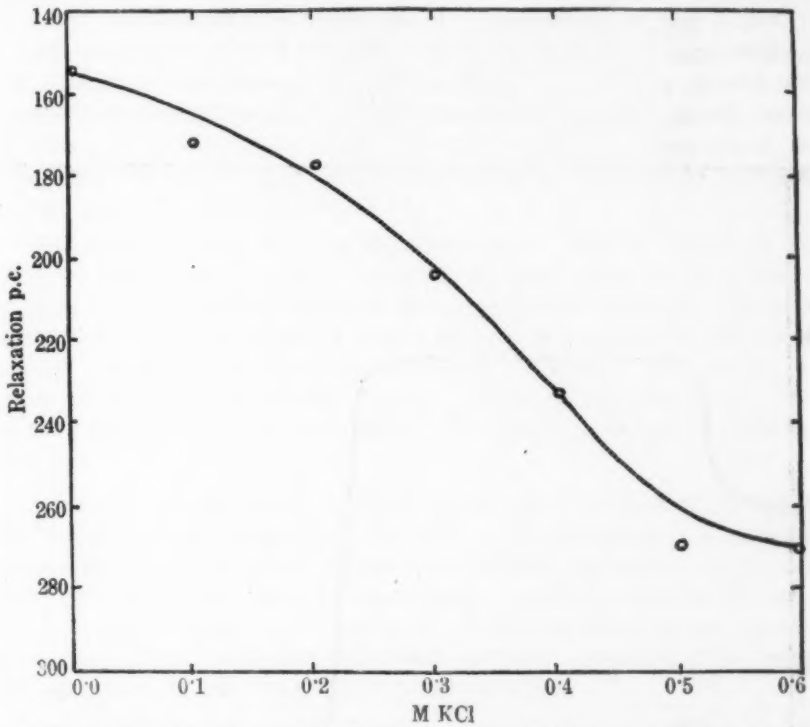

FIG. 2. Dog's stomach muscle. Effect of potassium chloride on contraction and relaxation. 24 hours' immersion as in Fig. 1.

KCl. Large concentrations of potassium chloride, 0·4 *M* KCl and upwards, upto 2 *M* KCl caused only relaxation varying from 200 to 340 p.c. Thus in majority of the experiments the effect of potassium chloride on the contractile mechanism of unstriated muscle resembles that on actomyosin in solution as shown by Szent Gyorgyi (1951), contraction of muscle corresponding to precipitation or superprecipitation and relaxation to dissolution or dissociation of actomyosin.

The addition of magnesium chloride (0·01 *M* $MgCl_2$) and adenosine-triphosphate (ATP), 0·05 p.c. and 0·02 *M* sodium pyrophosphate did not produce any significant difference in the results; ATP most likely decomposes. This apparently differs from the behaviour of actomyosin in solution, but it must be remembered that the muscle contains both these substances in places, just where they ought to be, so that the effect of these substances appeared when the concentration of potassium chloride was raised in steps of 0·02 *M* KCl (6 experiments). It was found in 4 experiments, that contraction occurred abruptly in about 0·04–0·06 *M* KCl, and relaxation was also abrupt, a difference in concentration of as little as 0·02 *M* KCl produced almost complete relaxation Fig. 3. This curve resembles that of

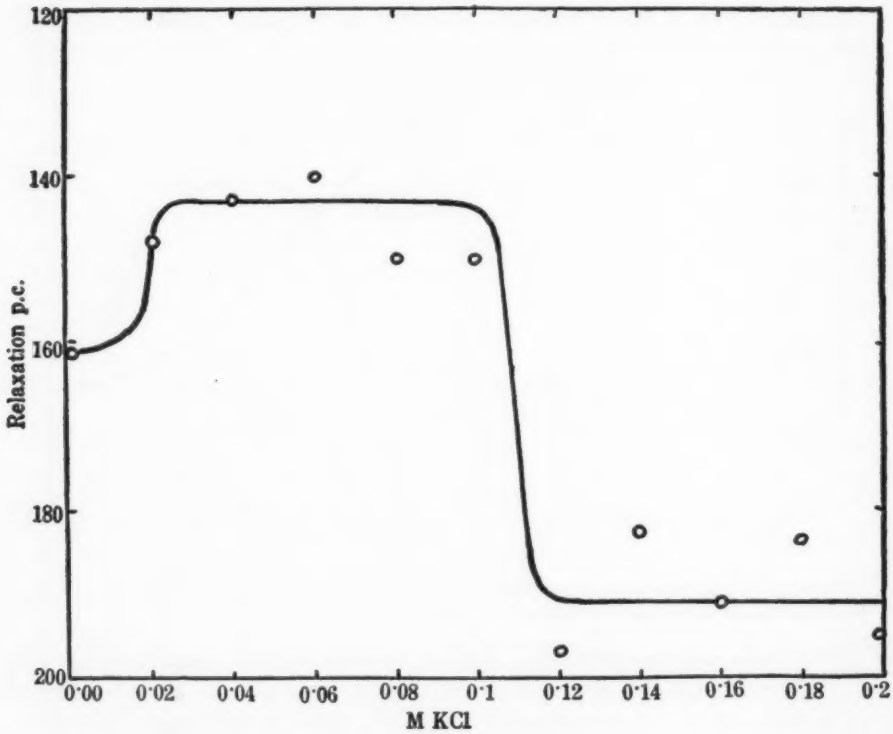

FIG. 3. Dog's stomach muscle. Effect of potassium chloride on contraction and relaxation. 24 hours' immersion as in Fig. 1.

superprecipitation of actomyosin and there can be hardly any doubt that there must be some close relationship between the superprecipitation of

actomyosin and contraction of muscle, contraction being due to association, and relaxation to dissociation of actomyosin.

The addition of calcium chloride, 0·01 *M* $CaCl_2$, to the potassium chloride solutions, did not produce any significant difference.

In potassium chloride solutions, unloaded muscles did not relax to any great extent (from 0 to 20 p.c.), though the loaded muscles relaxed enormously (Singh and Singh, 1949, 1950 *b*).

Effect of urea.—Urea is another group of substances that cause dissociation of actomyosin (Mommaerts, 1950). The action of urea on muscle is quite different from that of potassium chloride; urea causes marked active relaxation (Singh and Singh, 1949). The dissociation of actomyosin at the bonds which are dissolved by urea, is therefore responsible for active relaxation of unstriated muscle.

Potassium chloride acts on different bonds. This is shown by the following experiments: (1) After maximal relaxation of an unloaded muscle by urea, potassium chloride does not cause any further relaxation. (2) After maximal relaxation of a loaded muscle by urea, potassium chloride causes further relaxation. Loading or unloading, therefore, dissociates the action of urea and potassium chloride. Thiourea acts like urea. Thus we have the basis of active relaxation of unstriated muscle on the molecular level.

Effect of distilled water.—Distilled water causes marked active relaxation (Singh, 1944 *a*; Singh and Singh, 1949). This relaxation occurs due to dissociation of actomyosin at the same bonds which are acted upon by urea. This is shown by the following experiments: (1) After maximal relaxation of an unloaded muscle by distilled water, potassium chloride does not produce any further relaxation. (2) After maximal relaxation of a loaded muscle, potassium chloride causes further relaxation.

In a stretched muscle, distilled water may produce contraction, so that in these experiments, it appears that a substance diffuses out which is responsible for relaxation. This occurs more readily in the pyloric region of the frog's stomach, than in the cardiac region (Singh and Singh, 1950 *a*). As the pyloric region shows alactic tone, it appears that the association of actomyosin for production of alactic tone differs from that responsible for lactic tone. The effects of urea on a stretched muscle resemble those of distilled water.

Effect of other salts.—Potassium cyanide and sodium pyrophosphate act powerfully to cause dissociation of actomyosin (Szent Gyorgyi, 1951).

These substances (isotonic solutions) produce both active as well as passive relaxation. Alkalies also cause dissociation of actomyosin (Mommaerts, 1950); NaOH and Na_2CO_3 produce both active and passive relaxation (Singh and Singh, 1950 *b*). $NaHCO_3$ produces passive but not active relaxation. ATP (0·2–0·5 p.c.) has produced doubtful results; it probably decomposes during the course of the experiment. It has produced passive relaxation of muscle from the pyloric part of the frog's stomach in 2 out of 6 experiments and no effect in dog's stomach muscle. The pyloric part of the frog's and dog's stomach muscle shows alactic tone, which resembles the *rigor mortis* of striated muscle (Singh and Singh, 1951, 1952; Singh, 1952, 1953). As rigor is abolished in striated muscle by ATP (Erdos, 1943), it was expected that ATP would cause relaxation of unstriated muscle showing alactic tone. Sodium pyrophosphate, however, as mentioned above, relaxes the muscle. Guanidine and arginine produce passive but not active relaxation; these also cause dissociation of actomyosin (Mommaerts, 1950).

Effect of chloroform.—In saline, saturated with chloroform, the muscle becomes inexcitable. In its presence, the effects of potassium chloride are still produced (Fig. 1), so that these effects undoubtedly represent the reactions of the contractile mechanism. Chloroform makes the unwashed frog muscle behave similarly to washed frog muscle (Szent Gyorgyi, 1951).

Effect of potassium on the living physiological muscle.—The significance of the present experiments will be enhanced, if it can be shown that potassium produces similar effects on the contractile mechanism of muscle, in its normal physiological state. This can be shown with reasonable certainty on *Mytilus* unstriated muscle. The potassium content of this muscle is low (Singh, 1938 *a*). If this muscle is immersed in potassium-rich solutions, it rapidly gains potassium (Singh, 1944 *b*). If it is isotonically loaded and immersed in saline containing 0·4–0·6 *M* KCl, after initial contraction it markedly relaxes, and its viscosity, as shown by the rate of change of length, greatly diminishes (Singh, 1938 *b*, 1943 *b*). Further in such a high concentration of potassium chloride, the muscle becomes inexcitable.

The drop in viscosity of the muscle suggests that the actomyosin has dissociated during relaxation, as the viscosity of actomyosin solutions decreases during dissociation into actin and myosin. The action of potassium chloride is reversible and the muscle can be restored to its normal physiological state. If the muscle is immersed in saline containing 0·1 *M* KCl for 12 hours, it contracts. It is difficult to say whether its viscosity increases, since the rate of extension decreases, the rate of shortening increases (Singh, 1938 *b*).

Discussion

These experiments strongly support the views of Szent Gyorgyi on the contraction and relaxation of muscle. One kind of contraction and relaxation of unstriated muscle appears to be due to association and dissociation of actomyosin respectively. Further, they suggest that the dissociation of actomyosin is of two kinds; one produces passive and the other active relaxation. Superprecipitation of actomyosin does appear to correspond to contraction of muscle.

Unstriated muscle shows two kinds of tone, one resistant to asphyxia (alactic tone), and the other susceptible to asphyxia (twitch and lactic tone). The former is probably due to association of actomyosin by bonds which are dissolved by urea, and the latter by bonds which are dissolved by salts. The association of actomyosin in tonus of *Mytilus* muscle must be different, as the contractile mechanism of this muscle does not relax actively in cyanide, unlike that of frog and mammalian muscle, which relaxes actively.

Summary and Conclusions

1. Experiments have been performed on dying muscles. In such muscles, the excitatory system is destroyed and the substances act directly upon the contractile mechanism. The excitatory system was also rendered inoperative by chloroform.

2. In such muscles 0·1–0·3 *M* KCl produces contraction, and higher concentrations (0·4 to 2 *M*) produce passive relaxation. This suggests that contraction is due to association and relaxation to dissociation of actomyosin.

3. Urea and thiourea produce active relaxation. As urea is also known to produce dissociation of actomyosin, these experiments suggest that the dissociation of actomyosin is of two kinds, one producing active and the other passive relaxation.

4. Many salts which produce dissociation of actomyosin produce relaxation.

5. Distilled water causes active relaxation.

6. The effect of potassium chloride has been tested on living *Mytilus* muscle in normal physiological condition. These experiments also suggest that contraction is due to association and relaxation to dissociation of actomyosin.

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A MICRO-ELECTROPHORETIC STUDY OF HUMAN SERUM PROTEINS IN SMALL-POX

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The variation of the serum proteins under pathological conditions has been investigated by a large number of workers (Gutman, 1948; Luetscher, 1947). Among the methods available for the estimation of the serum proteins, the electrophoretic method is commonly employed on account of the ease with which the estimations can be carried out. The micro-electrophoretic methods have provided the additional advantage that the amount of material required is small and there is considerable saving of time in the analysis. Of the several diseases where serum analysis has been carried out, it is noticed that in two cases, *viz.*, nephrosis and some types of myelomatosis, the electrophoretic patterns are characteristic of the disease (Luetscher, 1947). In fact, they can be employed for diagnosing these diseases. In other cases, the ratio of the different protein fractions are found to vary considerably. Although electrophoretic analysis of human serum in various diseases has been reported, no systematic work seems to have been published in literature on serum proteins in small-pox. Tangredi and Robertaccio (1950) have reported on the total serum protein levels, at various stages of small-pox eruption, but they have not estimated the different protein constituents of the serum. The present study was, therefore, undertaken to carry out an electrophoretic analysis of the serum protein fractions, in cases of small-pox.

Experimental

The pathological sera were prepared from blood samples drawn from patients admitted to local isolation hospital. The normal sera were prepared from blood samples drawn from the staff and students of the Institute. The serum (1 c.c.) was diluted 4–5 times its volume with Michaelis (1931) buffer of pH 8·6, ionic strength $\mu = 0{\cdot}1$, dialysed against two or three changes of buffer for a period of 12 hours and finally dialysed against 250 c.c. of buffer for a period of 24 hours, employing cellophane bags at 0–4° C. After dialysis, the protein and buffer solutions were equilibrated for 30

minutes by surrounding with water at the room temperature and then deaerated for 30 minutes to remove the dissolved air. The electrophoretic analysis was carried out at room temperature employing Kern's micro-electrophoretic apparatus (Lotmar, 1951), using Jamin's interferometer to follow the protein boundary. The micro-electrophoretic cell had a capacity of 0·4 c.c. and was filled according to Longsworth's (1942) technique. In general, the electrophoresis was continued for about 60 minutes when the fastest moving boundary traversed the entire length of the cell. A potential gradient of 4–6 volts/cm. was employed for the electrophoresis. The initial and final boundaries were photographed by the optical device referred to above. The conductivity of the buffer was also determined for calculating the mobilities of the various protein fractions.

MOBILITY MEASUREMENT

The mobilities of the pathological and normal protein fractions were calculated by Longsworth's (1940) method making use of the descending limb of the channel. The results are given in Table I where the values of Armstrong *et al.* (1947) are also given for comparison.

TABLE I

Mobilities $\times 10^5$ (*sq. cm. volt.*$^{-1}$ *sec.*$^{-1}$) *of serum fractions in Michaelis buffer at pH* $= 8{\cdot}6$ *and ionic strength* $\mu = 0{\cdot}1$

Fraction	Normal		Pathological		Armstrong's values for normal serum
	22° C.	0° C. (calc.)	22° C.	0°C. (calc.)	0° C.
Albumin ..	11·20	5·98	11·31	6·03	5·95
α_1-globulin ..	9·90	5·29	10·02	5·35	4·85
α_2-globulin ..	8·00	4·27	8·50	4·54	3·85
β-globulin ..	5·43	2·90	5·18	2·77	2·83
γ-globulin ..	1·87	1·00	1·59	0·85	1·05

Since Michaelis buffer was employed in the present studies the δ and ϵ boundaries were not noticed and hence the initial boundary was taken as the reference boundary from which distances traversed by other fractions were measured (Kern's Manual, 1952). The mobilities were reduced to 0° C. by Johnson and Shooter's (1948) method. The values further indi-

cated that the mobilities of normal and pathological serum proteins did not differ to any marked extent. These observations were also in agreement with the recent results of Lippman *et al.* (1952).

Electrophoretic Patterns of Normal and Small-pox Sera

The interferometric patterns obtained for the ascending and the descending limbs of the channel for serum proteins under normal and pathological conditions are given in Fig. 1. In this pattern, each protein fraction shows a characteristic set of symmetrical fringes. Labhart and Staub's (1947) method has been employed for calculating the various serum fractions. The method consists of plotting a fixed distance as ordinate for each of the fringes at their positions and drawing the curve to join these points. The points of inflection represent the individual fractions, the relative concentrations of which can be read from the ordinate as shown in Fig. 2. The total protein has been determined by multiplying the number of fringes with the cell constant as indicated by Longsworth (1952). The total protein is also determined by the micro-Kjeldahl method in some cases to check up the cell constant.

Table II gives the results of normal and pathological sera. The normal values have been compared with values of Armstrong *et al.* (1947), Dole (1944), and Moore *et al.* (1949). The results for the normal serum obtained

TABLE II

Average values of the protein fractions in normal human serum and in the serum of persons infected with small-pox

Protein fraction	Normal human serum				Small-pox sera %
	Armstrong *et al.* %	Dole %	Moore *et al.* %	Our values %	
Albumin ..	59·0	63·4	55·4	60·4	40·8
α_1-globulin ..	5·7	4·8	7·8	5·2	10·0
α_2-globulin ..	9·3	7·6	8·1	10·6	15·1
β-globulin ..	14·3	12·7	18·4	12·1	14·4
γ-globulin ..	11·8	11·6	10·7	11·7	18·2

by us agree with the data of Armstrong *et al.* (1947). The electrophoretic data for nine samples of normal sera and twelve samples of small-pox sera are presented in Tables III and IV respectively.

TABLE III

Mobility ($u \times 10^5$) at 22° C. and distribution of protein fractions in normal human serum (Michaelis buffer of pH 8·6, ionic strength $\mu = 0·1$)

No.	Albumin		α_1-globulin		α_2-globulin		β-globulin		γ-globulin		Concentration of protein in cell g./100 c.c.	Total protein in serum g./100 c.c.
	%	Mobty.	%	Mobty.	%	Mobty.	%	Mobty.	%	Mobty.		
1	60·5	11·37	5·0	9·97	10·0	8·36	12·5	5·31	12·0	1·78	1·55	7·29
2	60·0	11·53	5·0	10·31	10·0	8·67	12·5	5·53	12·5	1·69	1·27	7·68
3	60·0	11·34	5·5	10·31	11·0	8·95	11·5	5·85	12·0	2·00	1·15	7·74
4	61·5	10·74	5·0	9·80	12·0	8·80	11·5	5·34	10·0	1·89	1·15	7·74
5	60·0	11·23	5·5	9·83	10·0	8·98	12·5	5·06	12·0	1·89	1·55	7·00
6	61·0	10·69	3·0	7·67	9·5	5·65	12·5	3·40	14·0	·96	4·77	7·77
7	65·0	10·58	4·5	7·34	7·5	5·65	12·5	3·39	10·0	·98	3·55	7·50
8*	65·0	13·09	3·5	10·80	11·0	8·84	8·0	5·84	12·5	2·03	1·15	7·74
9*	66·0	10·94	3·5	9·22	8·0	7·49	9·0	4·92	13·0	1·92	1·33	7·67

* Analysis carried out when $\mu = 0·05$.

TABLE IV

Mobility ($u \times 10^5$) at 22° C. and distribution of protein fractions in small-pox human serum (Michaelis buffer of pH 8·6, ionic strength $\mu = 0·1$)

No. and clinical status	Albumin		α_1-globulin		α_2-globulin		β-globulin		γ-globulin		Concentration of protein in cell g./100 c.c.	Total protein in serum g./100 c.c
	%	Mobty.	%	Mobty.	%	Mobty.	%	Mobty.	%	Mobty.		
1 FS ··	39·0	11·34	14·0	10·00	11·0	8·58	23·5	5·58	12·5	1·79	1·12	5·47
2 FM ··	48·0	11·11	9·5	10·02	5·5	8·44	17·5	5·11	19·0	1·60	1·60	5·22
3 MM··	44·5	10·64	10·0	9·71	17·0	8·36	13·0	5·11	15·5	1·76	1·24	4·96
4 MS ··	42·0	11·43	12·5	10·02	25·0	8·67	9·5	4·81	11·0	1·31	0·99	4·97
5 MS ··	30·5	11·33	13·0	10·02	20·0	8·79	15·5	5·68	21·0	1·43	0·99	4·97
6 MS ··	30·5	11·32	7·0	10·07	16·5	8·43	10·0	5·04	36·0	1·46	1·89	5·67
7 FS ··	37·5	11·15	6·0	9·68	23·0	8·08	10·5	4·99	23·5	1·62	1·39	5·58
8 FS ··	45·0	11·67	6·5	10·02	15·0	8·77	11·0	5·77	17·5	1·75	0·67	5·35
9 MM··	48·5	11·00	6·5	10·00	11·5	8·58	15·5	5·43	17·5	1·71	0·89	5·35
10 FM ··	43·5	11·44	12·5	10·07	8·0	8·08	21·5	5·24	14·5	1·70	0·80	5·10
11 FS ··	40·0	11·40	12·0	10·01	14·0	8·70	11·0	4·90	22·0	1·53	0·84	5·35
12 MS ··	24·5	11·30	12·0	10·00	10·0	8·09	16·5	4·99	37·0	1·50	0·75	5·98

FS — Female, severe case; FM — Female, mild case;
MM — Male, mild case; MS — Male, severe case.

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FIG. 1. Electrophoretic Interferometric pattern with Michaelis buffer

Normal human serum: A—Ascending limb; B—Descending limb

Smallpox human serum: C—Ascending limb; D—Descending limb

FIG. 2. Evaluation curve of the interferometric pattern of Fig. 1 B.

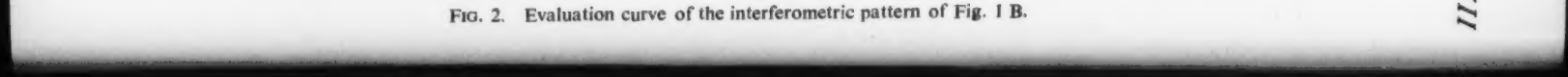

FIG. 2. Evaluation curve of the interferometric pattern of Fig. 1 B.

Discussion

Results of Table II indicate that small-pox is a case of hypo-proteinemia and hyperglobulinemia. The increase in γ-globulin under pathological condition is quite marked in all the cases studied, except in cases 1 and 4 (Table IV). It is also clear that all the globulins show a marked increase, while the albumin fraction shows the opposite effect. This phenomenon, as explained by Gutman (1948), may be due to the liver damage or febrile disease or the immunological response of the proteins. The stages of the disease have been classified as mild and severe on the basis of clinical information at the time of blood sampling. Employing Howe's (1921) technique, Datta and Chakravarty (1947) and Datta (1947) have carried out an extended study on plasma proteins in health and disease and have shown that in the diseases studied, there is a fall in albumin and an increase in the globulin contents of the serum. Our results for the total protein content in normal human sera confirm their data. Experiments on undiluted serum and at lower ionic strength did not reveal the presence of any new component in normal and pathological sera. The observations of Tangredi and Robertaccio (1950) on the total decrease in serum proteins in small-pox is in agreement with our studies.

Summary

An electrophoretic analysis of normal and small-pox human sera has been carried out employing Kern's micro-electrophoretic apparatus using Michaelis buffer of pH 8·6 and ionic strength $\mu = 0{\cdot}1$. The results indicate that the pathological condition brings about an increase in all the globulin fractions and a decrease in the albumin moiety.

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